

## A gene controlling biosynthesis of isoorientin, a compound in corn silks antibiotic to the corn earworm

N. W. Widstrom<sup>1</sup> & M. E. Snook<sup>2</sup>

<sup>1</sup>USDA-ARS, Insect Biology and Population Management Research Laboratory, Plant Resistance/Germplasm Enhancement Unit, P. O. Box 748, Tifton, GA 31793, USA (E-mail: nwidstro@tifton.cpes.peachnet.edu); <sup>2</sup>USDA-ARS, Russell Agricultural Research Center, Phytochemical Research Unit, P. O. Box 5677, College Station Road, Athens, GA 30613, USA

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### Abstract

The corn earworm (*Helicoverpa zea* Boddie) is an important pest of corn (*Zea mays* L.), and its larvae sometimes cause severe ear damage to hybrids grown in the southeastern United States. The antibiotic compound isoorientin is present in silks of some corn inbreds at a concentration that is harmful to corn earworm larvae. The inbred T218, which produces biologically active levels of this compound (>2.0% dry weight), was evaluated in hybrid combination with two other non-isoorientin producing inbreds to determine the mode of isoorientin inheritance in corn silks. Silk masses from individual ears of each parent, the F<sub>1</sub>, F<sub>2</sub>, first backcrosses, F<sub>3</sub> families and selfed BC<sub>1</sub> families were evaluated in 1994 and 1995 for isoorientin concentration. Reversed-phase high performance liquid chromatography (HPLC) was used to make chemical determinations. Segregation ratios in the F<sub>2</sub>, first backcross to T218, F<sub>3</sub> families and selfed BC<sub>1</sub> families were tested. The tests were conclusive in the identification of a single recessive gene controlling high isoorientin concentration in the silks of inbred T218. Some evidence for modifiers exists, in that there was a statistically nonsignificant trend for more plants than expected to occur in the low isoorientin concentration classes. Development of inbreds with a high concentration of the simply inherited isoorientin in their silks will add to the arsenal of compounds available in corn silks to combat damage to corn by corn earworm larvae.

### Introduction

Concerns about increased environmental pollutants due to chemical control of plant pests and pathogens in agriculture are being met with efforts to more fully use the natural defenses of plants against these destructive agents. There has been a renewed interest in reducing damage to corn, *Zea mays* L., by larvae of the corn earworm, *Helicoverpa zea* Boddie, using the natural chemical defenses in corn silks since identification of maysin, a C-glycosylflavone (Figure 1) found in corn silks with antibiotic activity against corn earworm larvae (Waiss et al., 1979). Other C-glycosylflavones, maysin analogues with antibiotic activity and lesser activity than maysin were soon identified (Elliger et al., 1980b). An effective dose rate of maysin was

determined for the corn earworm using procedures developed by Chan et al. (1978). Concentrations ranging from 1.5 to 2.0% dry weight were found to reduce larval growth by 50% (Waiss et al., 1979, 1981). Elliger et al. (1980a) found that the potency of larval growth inhibition was related directly to the number of hydroxyl groups attached to the phenyl (B) ring. (Figure 1).

A spectrophotometric method for quantifying maysin, using UV absorbance at 352 nm in methanol extracts, was developed by Waiss et al. (1979). This method was used by Widstrom et al. (1982, 1983), Henson et al. (1984), and Wiseman et al. (1985) in a series of experiments, but the resulting values were variable within and between years. Snook et al. (1989) developed a reversed-phase HPLC procedure that is

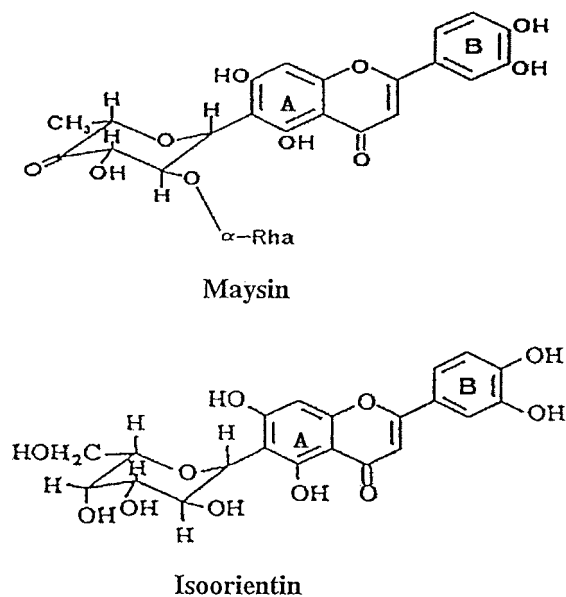


Figure 1. Chemical structures for maysin and isoorientin. Rha = rhamnose. Note that the sugar attached to the A ring is rhamnose for maysin, but glucose for isoorientin.

highly specific for maysin and separates interfering compounds from the analysis. The new technique made possible a clearer interpretation of genetic data (Widstrom et al., 1991) and resulted in significant correlations between the weights of larvae that fed on silks and HPLC maysin evaluations (Wiseman et al., 1992). The reversed-phase HPLC procedure is being used to obtain detailed genetic information on at least one inbred line, GT114 (Widstrom & Snook, 1994; Byrne et al., 1996) and for developing general information that facilitates efficient selection among other lines and hybrids (Widstrom & Snook, 1998).

A useful consequence from development of the reversed-phase HPLC procedure was the identification of several analogues and derivatives of maysin (Snook et al., 1993, 1995). One of these compounds is found in certain inbreds (notably T218) that has maysin concentrations that are effective (approximately 2.0% dry wt.) for manifesting some growth suppression of corn earworm larvae, but produces a very low larval weight when bioassayed (Wiseman et al., 1992). T218 however, produces several other peaks in its HPLC profile that are attributable to luteolins (Gueldner et al., 1991). The most prominent of these peaks was one produced by a compound first designated as 6-C-galactosyl-luteolin (Snook et al., 1994) but has more recently been shown to be 6'-C-glucosyl-luteolin, more commonly known as isoorientin (Figure 1) (unpublished).

This compound, in conjunction with its maysin concentration, is apparently responsible for lower larval weights than expected for maysin in T218. Derivatives of this compound were identified as constituents of corn silks by Levings & Stuber (1971) and similar compounds were reported as constituents of silks and tassel glumes (Ceska & Styles, 1972). Reid et al. (1992) reported that isoorientin is one of the major phenolic compounds present in silk extracts of corn inbreds they tested for resistance to *Fusarium* spp. The antibiotic activities of maysin and isoorientin against corn earworm are nearly equal because both are dihydroxylated on the phenyl (B) ring (Figure 1) (Elliger et al., 1980a).

The specific placement of isoorientin and maysin in the flavonoid pathway of maize is not known, except to say that they occur in that section of the pathway in which the C-glycosyl flavones are produced. This segment of the pathway is regulated by the *pl* locus (Byrne et al., 1996). They may also be controlled by other common genetic mechanisms. Our objective in this study, therefore was to investigate the inheritance of isoorientin in corn silks as a potentially important antibiotic compound that could be used in conjunction with maysin as a weapon against larvae of the corn earworm, and to seek additional clues regarding the genetic relationship between the two compounds.

## Materials and methods

The corn inbred T218 was chosen as the test organism to study the inheritance of isoorientin because it produces an average concentration of isoorientin greater than 2% by dry weight in its silks. This concentration of isoorientin combined with a maysin concentration of more than 2% (Table 1) severely inhibits the growth of first instar larvae of the corn earworm. T218 was crossed with two inbreds, L621 and N101, each having a negligible amount of antibiotic compounds in their silks (Table 1). These two non-antibiotic producing inbreds were chosen for crossing to maximize the probability that isoorientin produced by plants in hybrids or segregating populations would be attributable to T218.

Field plantings were made on 26 April 1994 and 25 April 1995 in rows that were 3 m long and 0.9 m wide with plants approximately 0.3 m apart within rows. Individual silks were collected at 3–5 days after they appeared from earshoots that had been covered to prevent pollination prior to sampling. The silks

Table 1. Distribution of individual plants of parent, F<sub>1</sub>, F<sub>2</sub>, and first backcross generations of maize crosses T218 × N101 and T218 × L621 for silk isoorientin concentrations and generation means for isoorientin and maysin

Cross	Generation	Number of plants							Isoorientin (% dry wt)	Maysin
T218 × N101	P <sub>1</sub>	15							2.02	2.83
	P <sub>2</sub>	15							0.00	0.18
	F <sub>1</sub>	15							0.01	2.05
	F <sub>2</sub>	64	3	1	3	1	8	0.23	1.78	
	BC <sub>1</sub>	14	2		1	2	3	1.07	2.06	
	BC <sub>2</sub>	40							0.03	0.86
	—	—	—	—	—	—	—	—		
T218 × L621	P <sub>1</sub>	1†	14						2.24	2.53
	P <sub>2</sub>	15							0.00	0.00
	F <sub>1</sub>	15							0.02	0.97
	F <sub>2</sub>	65	3	4	2	1	2	0.12	0.92	
	BC <sub>1</sub>	26			1	3	5	0.40	2.08	
	BC <sub>2</sub>	40							0.01	0.39
		≤0.15	0.25	0.45	0.65	0.85	1.05	>1.15		

Mid-class value for isoorientin concentration in silks (% dry weight).

† Assumed to be an outcross or result of seed mix.

were placed in a cold box, transported immediately to the laboratory, weighed, submerged in methanol, and placed in a freezer at  $-24^{\circ}\text{C}$  prior to shipment to the Richard Russell Research Center at Athens, Georgia where the chemical determinations were made by reversed-phase HPLC (Snook et al., 1989).

The 1994 test consisted of six populations 15 P<sub>1</sub>, P<sub>2</sub>, and F<sub>1</sub> plants, 80 F<sub>2</sub> and 40 plants each of BC<sub>1</sub> and BC<sub>2</sub>. Families of selfed F<sub>2</sub>s and first backcrosses were generated in the 1994–1995 winter nursery. Twenty-five F<sub>3</sub> families and 20 selfed BC<sub>1</sub> families for each of the crosses T218 × L621 and T218 × N101 were planted for evaluation in 1995. Sampling procedures for harvesting silks were identical to 1994 except that only 10 plants of each F<sub>3</sub> and selfed BC<sub>1</sub> family were evaluated.

The chemical data were recorded for individual plants and plotted to determine each population or family group distribution. The distributions of F<sub>2</sub>s and backcrosses were examined for segregation of high and low isoorientin concentration types, and a concentration of 0.15% was determined to be the point where minimal plant frequencies begin, if not a clear break, in the distributions of F<sub>2</sub>s and BC<sub>1</sub>s, separating high and low isoorientin classes.

Similar to 1994, distributions of individual plant values within F<sub>3</sub> and selfed BC<sub>1</sub> families were plotted to determine the class assignment for progenies of selfed plants. The Chi-square statistic was employed

to test the segregation ratios of individual plants in the F<sub>2</sub>, BC<sub>1</sub>, and of F<sub>3</sub> and selfed BC<sub>1</sub> families (Steel & Torrie, 1960).

## Results

Plants of inbred T218 consistently produce silks averaging more than 2.0% isoorientin and 2.5% maysin on a dry weight basis (Table 1). The range of values for isoorientin was 1.5 to 2.8% for 29 plants reported in the two experiments of this study. A single plant value for T218 of less than 0.1% (Table 1) in the T218 × L621 experiment was disregarded and assumed to be an outcrossed plant, a plant resulting from seed mix or some other source of experimental error. All F<sub>1</sub> plants in both experiments were similar to N101 or L621, in that their value for isoorientin was essentially zero, leading us to the obvious genetic hypothesis of recessive inheritance.

A slight tendency for plants in segregating generations to be skewed toward low isoorientin values existed, especially for the cross T218 × L621. The small excess of low isoorientin plants was not sufficient to produce significant deviation from a 3:1 ratio in the F<sub>2</sub>s and a 1:1 ratio in BC<sub>1</sub> generations for both crosses (Table 2). Eighteen plants of the T218 × N101 BC<sub>1</sub> had silks exceeding 1.15% isoorientin while only

Table 2. Tests of segregation ratios of plants from the F<sub>2</sub> and backcrosses to parent T218 of two maize hybrids for silk isoorientin concentration, assuming a single recessive gene for high isoorientin concentration

Cross	Generation	N	Low: High ratio		$\chi^2$ <sup>a</sup>
			Observed	Expected	
T218 × N101	F <sub>2</sub>	80	64:16	3:1	0.82
	BC <sub>1</sub>	40	14:26	1:1	3.02
T218 × L621	F <sub>2</sub>	77	65:12	3:1	2.16
	BC <sub>1</sub>	40	26:14	1:1	3.02

<sup>a</sup> Chi-square values were adjusted using Yates' correction for continuity (Steel & Torrie, 1960).

five exceeded that concentration in the T218 × L621 BC<sub>1</sub>.

A skewing effect toward the low isoorientin end of the distribution also occurred among F<sub>3</sub> families of the T218 × L621 cross (Table 3). Nine of 25 families were classified as producing only low isoorientin plants in this cross, again suggesting the existence of a modifying influence from the L621 side of the hybrid. The incidence of segregating families among selfed BC<sub>1</sub>s at nearly twice that of high isoorientin families gives weak evidence for the existence of modifiers in both non-recurrent parents. The skewing was insufficient to produce significant  $X^2$  values (Table 3).

Concentrations of maysin in the T218 parent were similar to those of isoorientin, but the inheritance patterns manifested in the F<sub>1</sub> and segregating generations were different. The average concentration of maysin in the F<sub>1</sub> of T218 × N101 (2.05%) (Table 1) indicated a near complete dominance level, while that in the F<sub>1</sub> of T218 × L621 (0.97%) suggested an additive type of genetic effect. Both seemed to be controlled by a different type of inheritance, different yet from the recessive gene of GT114 found in the cross GT119 × Gt114 by Widstrom & Snook (1994). The distribution of plants for maysin content in segregating generations (Table 1) gave no indication of single factor inheritance as with GT114. No further attempt was made to explain the inheritance of maysin content in the T218 crosses, except to note that it was greatly different from GT114, and seemingly independent of genes controlling isoorientin in T218.

## Discussion

The production of C-glycosyl flavones in the flavonoid pathway, as regulated by the *p1* locus, has been clearly outlined by Styles & Ceska (1981) and Byrne et al.

(1996). Whether maysin serves as a precursor of isoorientin or vice-versa, or whether they are produced by separate processes in that portion of the pathway is not indicated.

All of the germplasm sources we have tested that produce silk isoorientin also produce maysin, but many which produce maysin have no isoorientin in their silks. Since the *p1* locus regulates the conditions necessary for maysin production, it must also determine whether isoorientin can be synthesized, however, an additional controlling factor must exist that determines whether isoorientin will or will not be synthesized in addition to maysin. This suggests that isoorientin is synthesized either downstream from maysin in the flavonoid pathway or upstream in a separate branching of the pathway. The authors favor the latter because of the simpler structure of isoorientin with one glucose attached at the 6 carbon position of the A ring at the flavone structure versus maysin with two rhamnose sugars, one attached to the flavone structure at the same 6 carbon position of the A ring, and the other attached in tandem to the oxygen at the 2'' position of the first rhamnose sugar (Figure 1).

Modifying genetic factors that may be involved apparently have only minor impact on the isoorientin content. There is more evidence for the influence of modifiers in the T218 × L621 cross than in the T218 × N101 cross. The limited number of plants having high isoorientin in BC<sub>1</sub> of the T218 × L621 cross suggests that some genetic mechanism having L621 as its source may be preventing full expression of the trait. The important thing related to breeding for inbreds and hybrids with effective levels of antibiosis is that several genotypes are available in crosses similar to those reported here that have concentrations of maysin plus isoorientin in their silks elevated enough (maysin plus

Table 3. Distribution of segregation types for silk isoorientin concentration within F<sub>3</sub> and selfed BC<sub>1</sub> families derived from the maize hybrids T218 × N101 and T218 × L621 grown in 1995

Hybrid	Family type	Classification						$\chi^2$ <sup>a</sup>
		Observed			Expected ratio			
		high	Segregating	Low	High	Segregating	Low	
T218 × N101	F <sub>3</sub>	4	18	3	1	2	1	4.92
T218 × N101	Selfed BC <sub>1</sub>	7	13	–	1	1	–	1.25
T218 × L621	F <sub>3</sub>	2	14	9	1	2	1	4.28
T218 × L621	Selfed BC <sub>1</sub>	7	13	–	1	1	–	1.25

<sup>a</sup> Chi-square values are adjusted using Yates' correction for continuity (Steel & Torrie, 1960).

isoorientin  $\leq 4.0\%$ ) to be highly effective against corn earworm.

Our data demonstrate the presence of a single recessive gene controlling high isoorientin concentrations in the silks of T218. The gene is expressed independently of maysin concentration, and in conjunction with maysin produces plants that have a combined silk concentration of antibiotic compounds, more than sufficient to suppress feeding by corn earworm larvae.

Isoorientin is, however, only one of several compounds present in corn silks, and the relative importance of isoorientin compared and/or in conjunction with others will provide the basis for further study. The presence of  $>2.0\%$  isoorientin in T218 silks is known to be sufficient for inhibiting growth of the corn earworm larvae, and has the potential of being a major factor in contributing to the development of corn hybrids with high levels of resistance to the corn earworm. Adding isoorientin to the arsenal of other antibiotic compounds produced by silks, e.g. maysin, will increase the effectiveness of plant resistance to this pest.

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